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Full Length Research Paper

PCR-DGGE fingerprints of microbial successional changes during fermentation of cereal-legume weaning foods

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Phenotypic identification and monitoring of the dynamics of naturally occurring microbial community responsible for the spontaneous fermentation of different cereal-legume weaning blends was carried out. Enumeration using culture-dependent method showed that cell counts increased within the first 24 h with the highest total viable count of 1.2 x 10^{12} cfu g^{-1} in maize-legume (1A) blend. Yeast counts increased drastically and no enterobacteria were observed within the first 24 h. At all fermentation times, acidity increased within 48 h and lowest pH value (3.60) was reached in maize–based blend. Phenotypic identification revealed that the isolated bacteria belong to the genera *Bacillus* species, *Staphylococcus aureus*, and *Escherichia coli*. The yeast isolates were identified as *Saccharomyces cerevisiae, Saccharomyces* species and *Hansenula* species while *Lactobacillus plantarum* and *Pediococcus acidilactici* were the predominant lactic acid bacteria (LAB). The analysis of the denaturing gradient gel electrophoresis (DGGE) pattern obtained with bacterial and LAB primers targeting the V3 region of the 16S rDNA genes clearly demonstrated that there was a major shift in the community structure within the first 24 h.

Key words: Successional changes, microbial communities, fermented weaning foods, PCR-DGGE.

INTRODUCTION

Spontaneous lactic acid fermentation of cereal products is the cheapest method of safely preserving weaning food, the contamination of which is a major cause of disease and associated malnutrition (FAO/WHO, 1997). During the fermentation process, a succession of naturally occurring microorganisms results in a population dominated by lactic acid bacteria (Steinkraus et al., 1983; Hounhouigan et al., 1993b; Johansson et al., 1995). In order to demonstrate the role of these organisms in the fermented products, it is essential to quantify the predominating groups of organisms and to investigate the dynamics of the overall community. In addition, the final quality as well as the storage of the product strongly relies on the way the fermentation was carried out.

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Many of the classical food microbiological methods used in the past were culture-based, with microorganisms grown on agar plates and detected through biochemical identification. Studies have shown that phenotypic identification of fermentative microflora is time-consuming and often problematic due to ambiguous biochemical or physiological traits (Hugas et al., 1993; Sierra et al., 1995). Moreover, when biodiversity is studied by conventional techniques, such as cultivation of bacteria on solid media, the results are quite biased because majority of microorganisms are not culturable using standard techniques (Amann et al., 1995). Genetic-based diagnostic and identification systems can greatly enhance the specificity, sensitivity and speed of microbial testing. The more precise bacterial identification techniques using taxonomic and discriminating methods include biochemical tests, 16S ribosomal DNA (rDNA) sequencing, sodium dodecylsulfate – polyacrylamide gel electrophoresis of proteins (Pot and Janssens, 1993), randomly amplified

polymorphic DNA (RAPD) finger printing (Tailliez et al., 1996), and Fourier transform infrared spectroscopy (Amiel et al., 2000). However, all these methods are labour intensive and time consuming.

Recently, more rapid molecular methods have been developed to analyze diversity within bacterial communities (Øvreas and Torsvik, 1998). These methods are based on direct analysis of DNA in the environment and do not require cell cultivation. They include single – stranded conformational polymorphism analysis (Lee et al., 1996), denaturing gradient gel electrophoresis (DGGE) and temporal temperature gel electrophoresis (TTGE) (Muyzer et al., 1993). All of these approaches involve extraction of nucleic acids (DNA or RNA), amplification of genes encoding 16S rRNA, and analysis of PCR products by a genetic fingerprinting technique (Muyzer, 1999). Furthermore, a recent evolution in the direct amplification and analysis of ribosomal RNA genes is the use of specific primers, which allows the amplification and analysis of 16S rRNA genes of defined groups within a complex microbial community. The analysis of this group – specific PCR fragments on a DGGE gel provides a valuable tool for monitoring the structure and dynamics of microbial populations over time and under the influence of environmental changes (Boon et al., 2002). These approaches are attractive since they enable detection of individual species as well as overall profiling of community structure changes with time.

In order to study the ecology of fermented food, it is important to know the structure of the microbial community in it and to identify the physiologically active organisms. The objectives of this study were to characterize naturally occurring microorganisms in fermented cereal-legume blends phenotypically, and to monitor the dynamics of the microbial community responsible for the natural/ spontaneous fermentation of the cereal-legume weaning blends using PCR-DGGE.

MATERIALS AND METHODS

Sample collection

The cereals, DMR-ESR-Y(maize) and cowpea (Ife-Brown) used were collected from the Institute of Agricultural Research and Training (I. A. R. and T.), Ibadan, Nigeria while KSV-15 (sorghum) and millet were obtained from the Institute of Agricultural Research (I. A. R.), Zaria, Nigeria in clean sterile polyethylene bags and kept in the refrigerator until use.

Sample treatment and processing

Manual sorting and winnowing of the grains to remove stones, debris and defective seeds were carried out. The grains were weighed (≈ 500 g), cleaned and steeped in distilled water for 24 h at room temperature. The soaked grains were later germinated in a stainless tray lined with wet filter paper in an air circulating incubator at 30°C for 24 and 48 h for legume and cereals, respectively.

Germinated seeds were separated from non–germinated seeds. The rootlets of the malted cereals were removed, thoroughly washed with distilled water and oven dried at 60°C for 24 h. For the legumes, the germinated seeds were dehulled, washed and oven dried at 60°C for 36 – 48 h. The germinated and dried cereal and legume samples were separately milled, sieved to obtain a fine flour (90% passing through a 300 nm pore sieve), packed in sterile bags before storage at 4°C until use. The cereal-legume blends were formulated in ratios 70:30 (cereal-cowpea) (Malleshi et al., 1989).

Fermentation and sampling

The different flour combinations were mixed with sterile milli Q water at a concentration of 30% (w/v) (Livingstone et al., 1993). Spontaneous fermentation was allowed to proceed at 30°C and sampled 24 h for 3 days with initial sampling at 0 h.The sample portion taken for molecular analysis was kept at -20°C until use while the portion for microbial enumeration and pH determination were processed immediately.

Microbiological analysis

The fermented blend samples were subjected to microbiological analysis to monitor the dynamic changes in the population in the cereal–legume blends fermentation. Ten grammes (10 g) of each blend was homogenized in 90 ml, ¼ strength Ringer Solution. Serial dilution was done with sterile milli Q water and 1 ml of the appropriate dilutions was mixed with molten agar and pour–plated in duplicates. The media used for microbial enumeration were Plate count agar (Oxoid) for estimation of total viable bacteria incubated for 24 h at 30°C; aerobic bacteria (Nutrient agar); for total lactic acid bacteria (LAB) (MRS agar, Oxoid) incubated at 30°C for 48 h in anaerobic jars with Anaerogen (Oxoid, Basingstoke, Hampshire, England), Violet Red Bile Agar (Difco) was used for total enterobacteria at 30°C for 48 h, and yeasts and moulds counts on Malt extract agar (oxoid) containing 0.5 mg/l streptomycin sulphate (sigma) incubated at 30°C for 3 – 5 days.

Microbiological counts were made after incubation and counts were expressed as $\log_{10}$ cfu per gram. Distinct colonies were randomly picked and purified twice by streaking on the same growth or culture medium. Pure cultures were grown on agar slants at 30°C for 24 h and kept at 4°C for further use.

Identification and characterization tests

Morphological, physiological and biochemical tests were carried out on all the isolated organisms and identified using Bergey's Manual of Systematic Bacteriology (Sneath et al., 1994).

pH measurements was carried out using pH meter (Hanna Instrument HI 8521). Titratable acidity was determined using the Kramer and Twigg (1970) formular:

$$\text{Lactic acid (g/100g)} = \frac{\text{Vol. of NaOH}}{\text{Vol. of sample}} \times 0.9$$

DNA extraction and PCR amplification

Total bacterial DNA was extracted from different fermented blends by the modified method described by Ampe et al. (1999a). The quality of the DNA extracts was routinely checked by using 1% agarose - 1 X TAE gel. Different regions of the 16S rDNA of the total bacterial community was amplified with the universal primer (Muyzer et al., 1993) while the total lactic acid bacteria community DNA was amplified with primers gc 338 f and 518 r spanning the V3 region of the 16S ribosomal DNA (Øvreas et al., 1997) as described by Ampe et al. (1999). Aliquots (5 µl) of the amplification products were analysed by electrophoresis in 1% agarose – 1X TAE gels.

Denaturing gradient gel electrophoresis analysis

The PCR products were then analyzed by denaturing gradient gel electrophoresis (DGGE) using a Bio-Rad D code apparatus and the procedure first described by Muyzer et al. (1993). Electrophoresis was performed in a 6 and 8% (w/v) polyacrylamide gels with 1X TAE buffer diluted from 50 XTAE buffer (40 Mm Tris base, 20 Mm glacial acetic acid, and 1 Mm EDTA) for total bacterial community and total lactic acid bacteria community, respectively. Two denaturant gradients were used for optimal separation of the products; one from 30 to 60% urea–formamide gradient for gc 338 f-518 r primer and the other universal primer from 40 to 60% urea–formamide gradient increasing in the direction of electrophoresis. Electrophoresis buffer (1XTAE) was maintained at 60^{o}C. The gels were electrophoresed to a constant voltage of 200 V for 5 h for total bacterial community (Muyzer et al*.,* 1993), and for 10 min at 20 V and then 3 h at 200 V for lactic acid bacteria community (Ampe et al., 1999a). Gels were then stained with silver staining, scanned and analyzed with the Quantity One software package (Bio-Rad, Richmond, California).

RESULTS AND DISCUSSION

The results obtained by the traditional enumeration of microorganisms with five different culture media during the 72 h fermentation time are shown in Figure 1. In general, for all fermentation, cell counts increased drastically within the first 24 h. The total microbial concentration on PCA increased within the first 48 h and later decreased by 72 h. The highest total viable count was observed in maize-based blend (1.2 x 10^{12} cfu/g) while the least count of 3.95 x 10^{7}cfu/g was recorded in sorghum-based blend by 48 h of fermentation. The traditional plating results showed that the microbiological changes occurring in the fermenting blends (Figures 1a-f) were mainly characterized by initial increase in total bacteria count, LAB population and yeast count. The number of aerobic bacteria (culturable) count increased within the first 24 h of fermentation except in maize-based blends, which increased from 10^{6} to 10^{10} cfu/g within 48 h of fermentation. This observation was similar to those reported by earlier workers (Sanni et al*.,* 1994; Wakil et al*.,* 2004; Michodjehoun-Mestres et al*.,* 2005). Apart from the flora present on the surface of the grains, microbial flora may have also developed during milling and malting processes, thus explaining the higher initial culturable count in the formulated weaning blends. The lactic acid bacteria count on MRS agar was close to those on PCA except that it decreased after 24 h. Yeast counts on MEA increased drastically within the first 24 h and the highest count of 5.8 x 10^{11} cfu/g was recorded in maize-based blend. The total lactic count, which includes both streptococci and lactobacilli, was higher than the total culturable (viable) count and yeast count. These results were in accordance with the findings of Chavan and Kadam (1989) while the dominance of lactic acid bacteria in spontaneous fermentation of cereals has been reported by some workers (Fields et al*.,* 1981; Odunfa and Adeyele 1985; Halm et al*.,* 1993; Olukoya et al., 1993). A concomitant increase in lactic acid bacteria and yeast counts was observed during the spontaneous fermentation of the cereal-legume weaning blends, an association that has been noted in several cereal foods (Wood, 1981; Adegoke and Babalola, 1988; Halm et al*.,* 1993; Hounhouigan et al*.,* 1993a – c; Michodjehoun-Mestres et al., 2005). Report has also shown that the development of lactic acid bacteria is stimulated by yeast, which provides soluble nitrogen compounds and other growth factors (Nout, 1991).

No enterobacteria were observed after 24 h on VRBGA medium. The inability to detect enterobacteriaceae in the fermented formulated blends while those present in the unfermented blends disappeared by 24 h fermentation time agreed with the observations of Usha and Chandra (1997), Wakil et al. (2004) and Michodjehoun-Mestres et al. (2005). The observed decrease that culminated in total disappearance in the enterics population also paralleled the decrease in pH, which was in accordance with the death kinetic of enterobacteriaceae due to low pH (<4.5) in similar fermented material (Nout et al*.,* 1989; Hounhouigan et al., 1993c).

In general, acidity increased within the first 48 h and the lowest pH value (3.60) was reached in maize-based blend (1A) while the highest value of 3.98 was observed in millet-based (6A) blend. The decrease in pH and increase in titratable acidity up to 48 h and further increase and decrease at 72 h in pH and titratable acidity respectively may be attributed to inhibition of the growth of the microbial population at pH below 4.3. Such a decrease in pH and increase in acidity due to microbial activity has been well documented in cereals, millets and cereal-pulse mixtures fermented with endogenous grain microflora or with pure cultures (Aliya and Geervani, 1981; Achi, 1990; Khetarpaul and Chauhan, 1990, 1992). The observed increase in titratable acidity could be due to dominance of the environment by lactic acid bacteria, which degrade carbohydrates result in acidification. These observations are in agreement with earlier studies by Nout et al. (1989) and Ariahu and co-workers (1999). High titratable acidity was reported to reduce incidence of diarrhoea in infants consuming fermented cereal porridge (Mensah et al., 1990). The cowpea-fortified blends will then have two important attributes, such as antimicrobial properties and high protein content.

Phenotypic characters have frequently been used for bacterial characterization and are the basis for numerical taxonomy (Vandamme et al., 1996). The total culturable (aerobic) bacterial isolated in this study were identified based on the colonial, microscopic and biochemical characteristics of the isolates. These methods permit the presumptive identification of bacterial isolates to a high level (Varnam and Evans, 1991). The aerobic bacteria isolated on Nutrient agar (NA) and plate count agar (PCA) were thus identified as *S. aureus, B. subtilis, Bacillus* species, *Enterococcus* species and *E. coli.* The yeast isolates were identified as *Saccharomyces cerevisiae, Saccharomyces* species and *Hansenula* species.

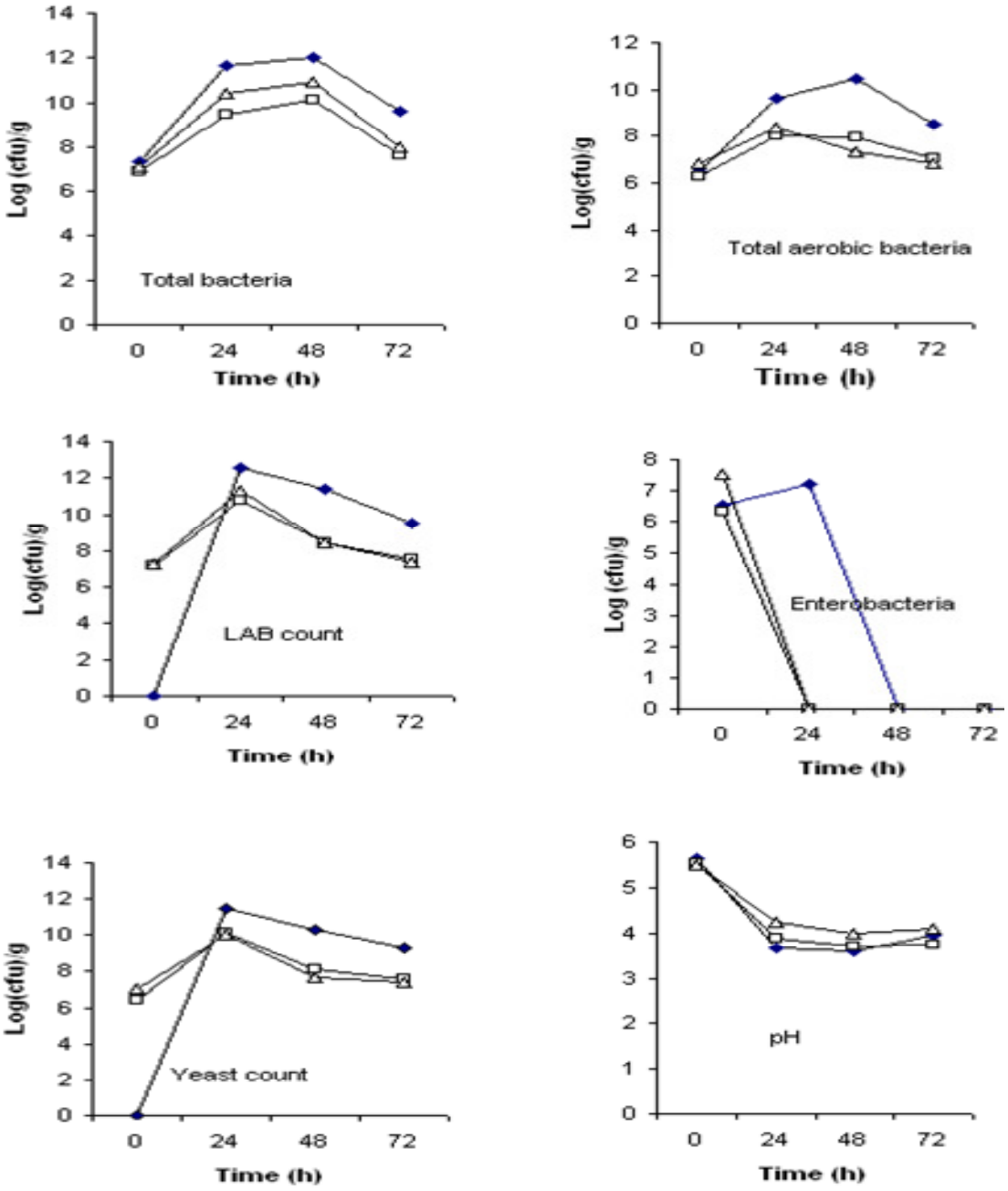

Figure 1. Microbial count during fermentation of cereal-legume blends using culture media: -□-sorghum-based; -ð-millet-based; -♦-maize-based.

The traditional plating results showed that the total culturable LAB populations in the fermented formulated weaning blends consisted of *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus* strains. Previous studies showed that naturally-fermented cereal-based African foods are dominated by *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus reuteri, Leuconostoc mesenteroides, Pediococcus pentosaceus* and *Lactococcus lactis* strains (Oyewole, 1995; Rombouts and Nout, 1995; Nuraida et al., 1995). According to Sharpe (1979), the ability to produce acid from carbohydrate is used routinely to differentiate and identify species of *Lactobacillus* as well as other lactic acid bacteria. The sugar fermentation patterns of the LAB isolates in this study conformed to this finding.

L. plantarum and *Pediococcus acidilactici* were the predominant lactic acid bacteria. The observed predominance of *L. plantarum* was also in agreement with the findings of other authors (Olsen et al., 1995; Usha and Chandra, 1997), so also the *Pediococcus* species (Usha

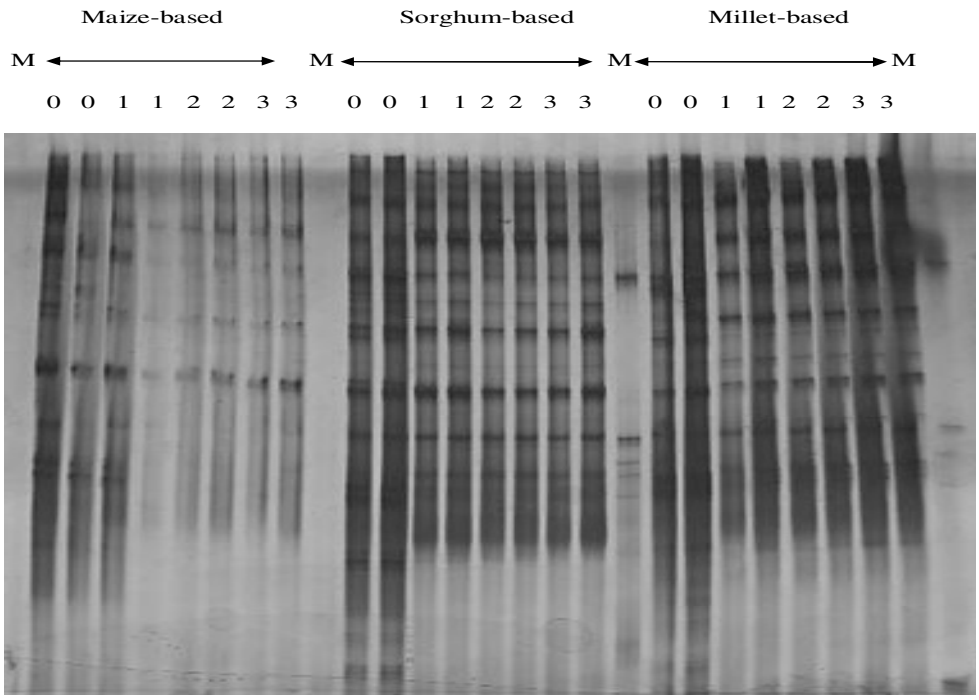

Figure 2. DGGE analysis of PCR- amplified 16S rDNA fragment for total bacterial community from cereal-legume weaning blends. 0, Unfermented blends; 1, 24 h fermented blend; 2, 48 h fermented blend and 3, 72 h fermented blend.

and Chandra, 1997).

Community fingerprinting by PCR-DGGE

The microbial population dynamics monitored by DGGE for total bacterial community profile and total lactic acid bacterial community profile was as shown in Figures 2 and 3, respectively. There were shifts in total bacterial and LAB communities' composition at different time intervals, and the shifts in community composition occurred in different patterns in all the three cereal-based weaning samples (Figures 2 and 3). This result suggests that the shifts in microbial population (some bands becoming dominant or recessive and new bands being formed) might have been due to cereal type and not necessarily as a result of increased fermentation time. This observation was similar to the findings reported by Omar and Ampe (2000), that the shift in the microbial community structure of fermented maize during pozol production coincided with the sampling point (whole, centre or periphery) which was also found to coincide with the development of *L. fermentum.* Furthermore, Ampe and Miambi (2001) reported differences between the microbial communities developed during indigenous maize fermentation for the production of Ogi, poto-poto and pozol, and reported that the differences are likely to be due to the differences in the processing methods and not fermentation time.

DGGE fingerprinting also allows comparison of total bacterial communities of the different samples through pattern analysis. An increase in number of bands was observed in the DGGE profile for total bacterial communities indicating an increase in total bacterial species, an observation similar to that of Santegoeds et al. (1996). The highest number of band with high band intensities was observed in millet-based blends, implying an increased number of predominating species of the microbial communities. This might be due to the high protein quality and mineral contents of pearl millet as reported by Khetarpaul and Chauhan (1989), FAO (1995) and Makokha et al. (2002).

Thus, the use of molecular method by the direct analysis of DNA shows that fermentation resulted in considerable reduction in species evenness with time in all the cereal samples. This means the dominance of a specific group of organisms (which are likely to be lactic acid bacteria). This conforms to the traditional (phenotypic) result that revealed the dominance of lactic acid bacteria with increased fermentation time. However, the observed LAB DGGE band intensities did not correlate with LAB concentration obtained by plating on MRS agar because the intensities increased with fermentation time. This result confirms the unsuitability of MRS medium for the cultivation or growing of lactic acid bacteria, an observation similar to that of Ampe and co-workers (1999b) and Ercolini et al. (2003).

In addition, despite the differences observed between

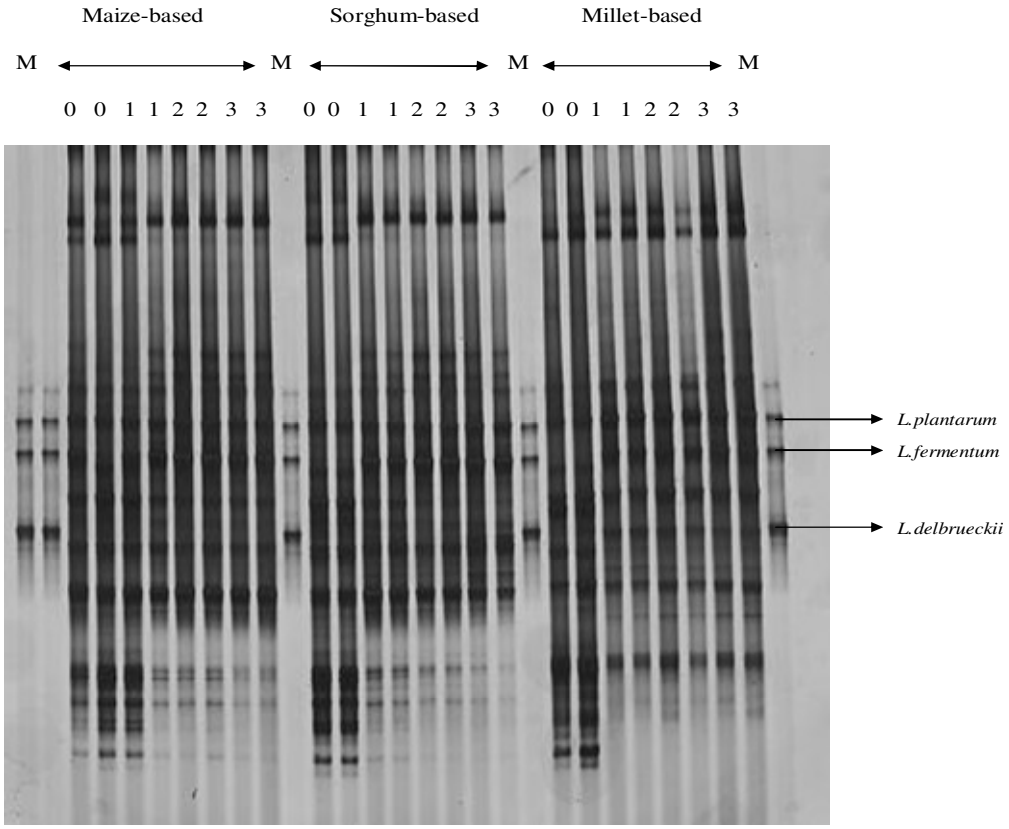

Figure 3. DGGE analysis of PCR- amplified 16S rDNA fragment for total Lactic acid bacterial community from cereal-legume weaning blends. 0, unfermented blends; 1, 24 h fermented blend; 2, 48 h fermented blend and 3, 72 h fermented blend.

the DGGE fingerprints of the different cereal – legume blends, some bands were present in most blends. Three of these widely distributed bands could be assigned to LAB species, by comparison of the migration distance of their PCR amplicons in DGGE gels with those of reference strains (Figure 3) as suggested by Meroth et al. (2003) and which is also in accordance with the findings of Ampe and Miambi (2000), and Walter et al. (2001). These bands correspond to *L. plantarum*, *L. fermentum* and *Lactobacillus delbrueckii*.

One of the important questions in the study of spontaneous fermentation is to determine whether it is the substrate, or the food matrix that is driving the process, or whether external conditions such as pH, oxygen and water activity are more important in the selection of the active microflora. Hence, the similarity between the DGGE pattern of the total bacterial community profile and that of the total lactic acid bacterial community profile were evaluated using Unweighted indexes only since weighted indexes has been reported not to always be in agreement with true quantification techniques of microbial taxons as a result of preferential amplification. For the evaluation of the effect of fermentation time on total bacterial and total lactic acid bacterial communities, UPGMA dendrograms were constructed using composite samples [replicate samples of the different cereals (maize, sorghum and millet) pooled together]. Results show a significant difference ($P<0.05$) between the total microbial communities of maize-sorghum- and millet- based blends. Dendograms are grouped in 4 distinct clusters (Figure 4). Each cluster corresponded to the different cereal-based used except cluster 4 and there was no replicate variability in duplicate samples of most blends at most time intervals. Cluster 1 contained millet-legume samples, cluster 2 contained sorghum-based samples, cluster 3, the maize-based samples while cluster 4 contained unfermented millet-based and sorghum-based samples. The observed differences between the microbial communities are likely to be due to the differences in the cereal-type (substrate) since each cluster belongs to a specific cereal-type while the fourth cluster suggested that there was no difference in the microbial community of unfermented cereal-blends irrespective of their type.

The similarity between the DGGE patterns of the lactic acid bacteria community revealed 3 distinct clusters (Figure 5). Here, fermentation time seems to have little effect on the microbial profile. Cluster 1 contained fermented millet-based samples with 97% similarity, cluster 2 non-fermented samples of the three (3) cereal-based

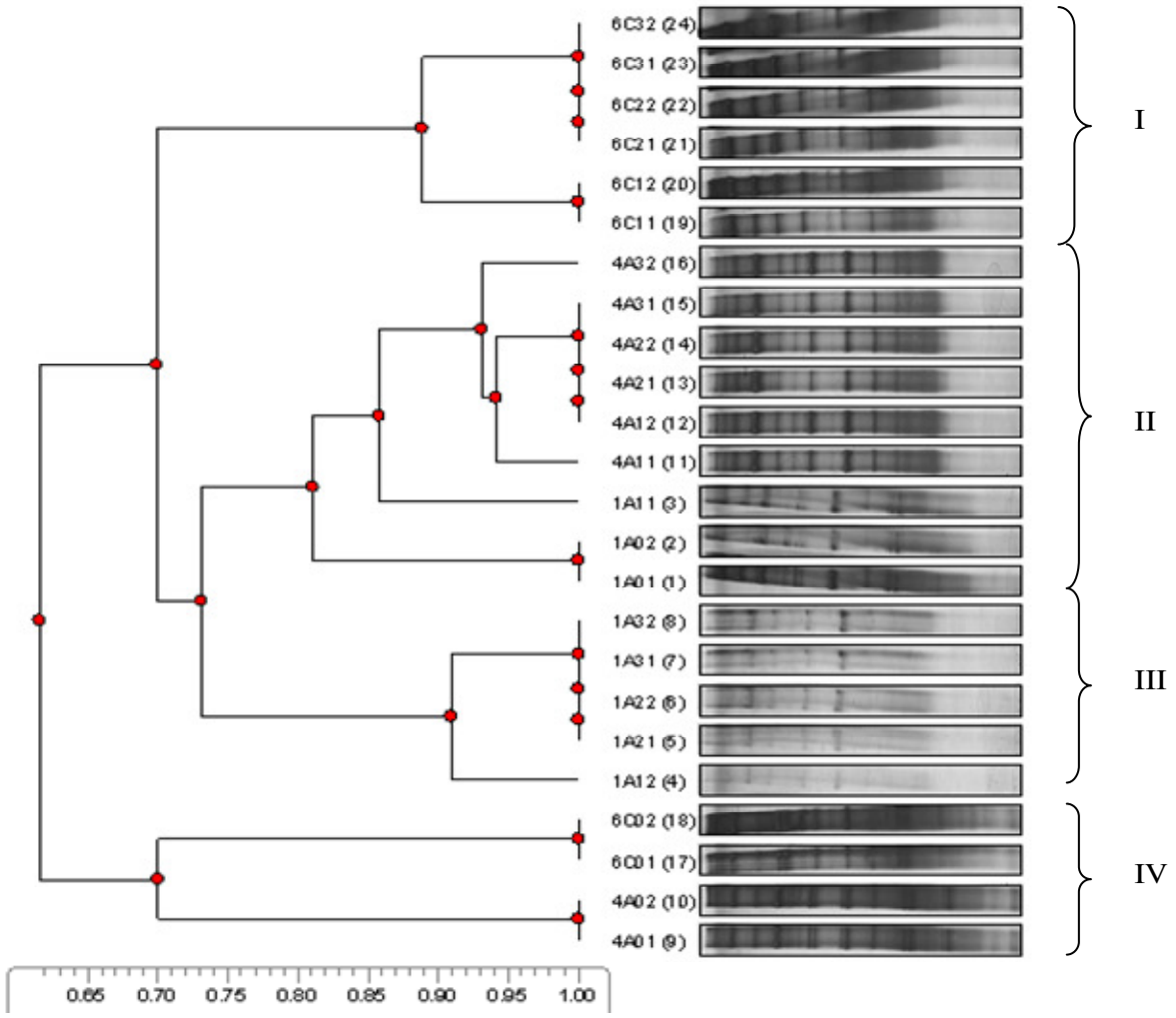

Figure 4. UPGMA dendrogram of DGGE profiles of amplified 16S rDNA from fermented cereal-legume blends for total bacterial community generated using Phoretix ID advanced analysis package.

samples with about 80% similarity and cluster 3 of about 90% similarity consisted of fermented samples of both maize- and sorghum-based samples. However, the total lactic acid bacteria species diversity among the fermented blends was significantly different ($P<0.05$). The unfermented blends formed a separate cluster (2) irrespective of the cereal type while there was a comparatively lower similarity between the total LAB community profile at different fermentation time in maize – and sorghum-based blends (Cluster 3). DGGE has been used to monitor the microbial dynamics during production of the Mexican fermented maize dough pozol (Ampe et al., 1999; Omar and Ampe, 2000) and to monitor the dynamic changes during wine fermentation (Cocolin et al., 2000). By applying the method to the spontaneous fermentation of cereal-legume weaning blends, we were able to determine that *L. plantaraum*, *L. fermentum* and *L.delbrueckii* were the predominant bacterial based on their migration, and were the organisms responsible for the nutritional and microbiological changes observed during natural fermentation of cereal based weaning foods. Furthermore, the use of PCR-DGGE to monitor the dynamics of the fermentation process revealed that bacterial community changes is more determined by the sample typed than by the fermentation time.

From the result, the microbial enumeration on different media can be compared to a large extent with the molecular approach but the molecular approach was advantageous in its specificity and rapidity. In conclusion, the shift in microbial population was found to be determined by the type of cereal, and to a lesser extent, the fermentation time, and the major shift was observed during the first 24 h.

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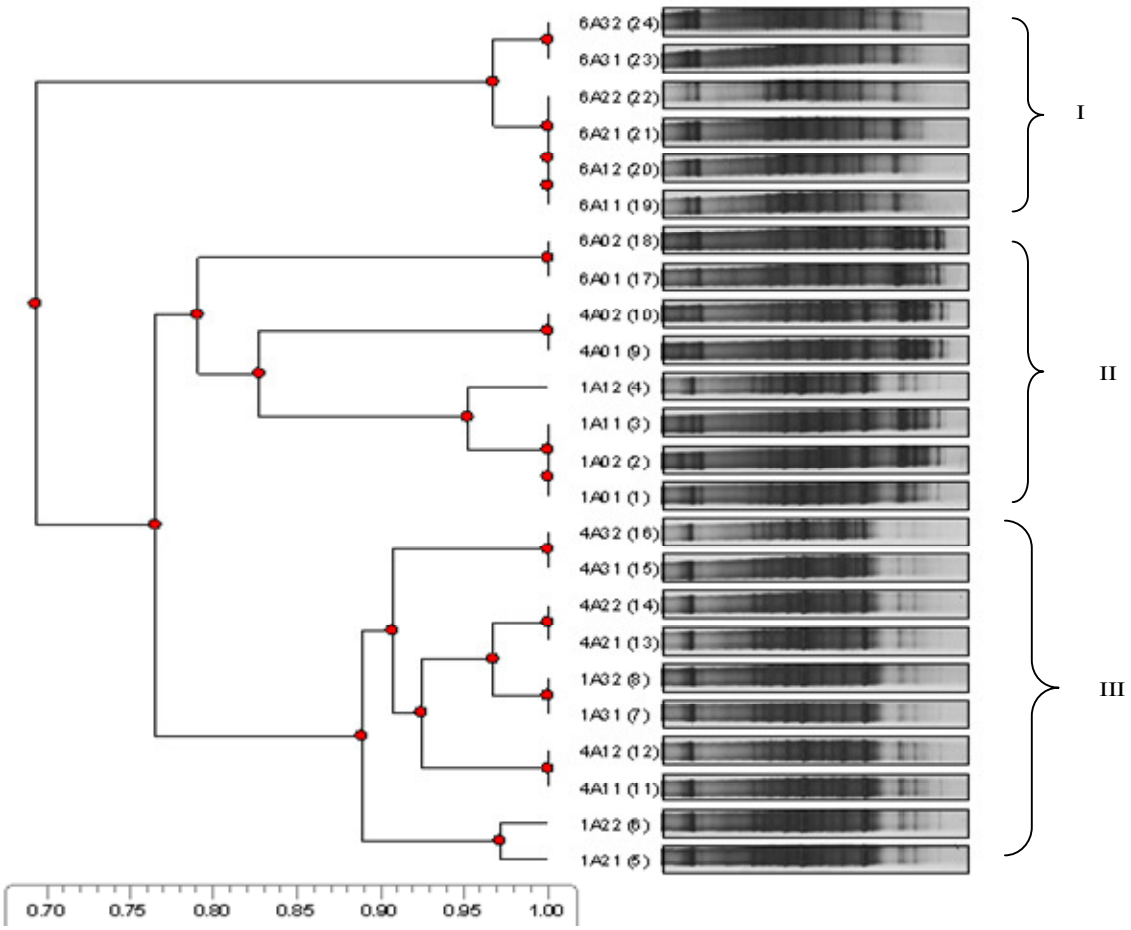

Figure 5. UPGMA dendrogram from DGGE profiles of amplified 16S rDNA from fermented cereal-legume blends for total lactic acid bacterial community generated using Phoretix ID advanced analysis package.

Training and Development Programme, University of Ibadan, Ibadan and the work was carried out at the Department of Biological sciences, University of Essex, Colchester, United Kingdom.

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